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THE USE OF THYMIDINE IN STUDIES WITH MAMMALIAN CELL CULTURES

II. The action of thymidine on the growth, metabolism, and morphology of HeLa S3 Cells

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by

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During the last few years, concern over radiation effects of tritium-labeled thymidine (H^3TdR) has permitted C^{14}-thymidine (C^{14}TdR) to be considered as practically free of hazards in radiobiologic and other metabolic studies on deoxyribonucleic acid (DNA). McQuade and Friedkin (1) have shown that the radiations emanating from this material can cause genetic damage, but little further attention has been given to its possible "pitfalls". It is the purpose of this communication to point out dangers in the use of C^{14}TdR in metabolic studies, especially with cell cultures. The results to be presented describe effects of thymidine that are due, not to its radioactivity, but to the fact that the inherent low specific activity of the C^{14} compound requires high concentrations that cause metabolic imbalances and disturbances in growth of cultured mammalian cells.

METHODS AND MATERIALS

To determine the effect of thymidine (or another nucleoside) on growth, HeLa S3 cells were inoculated into Leighton tubes at about 10^4 cells/tube. After 3 days incubation in Eagles' medium, sterile solutions of thymidine or other nucleosides (Nutritional Biochemicals Corp.), concentrated so that their addition did not appreciably expand the volume of the medium, were added to give the concentrations indicated under Results. Controls consisted of tubes to which an amount of sterile water, equal to the largest volume used for solutions of the nucleosides, was used. After three days further incubation, the cells in each tube were trypsinized with 0.5 ml 10 per cent trypsin solution (Microbiological Associates, Inc.) for ten minutes; 0.5 ml of Eagles' medium was added to each tube, and after

[illegible], the cells were counted in a hemocytometer, using the method for enumerating white blood cells.

Another kind of experiment was performed to check on the growth inhibitory action of thymidine. HeLa S3 cells were inoculated into petri dishes and allowed to incubate in Eagles' medium in an atmosphere of 5% CO_2 95% air for 18 hours. To each of six plates was then added a concentrated solution of thymidine to make 10 µg/ml. Similarly, six plates each were made to contain 20 µg/ml, 50 µg/ml, and 100 µg/ml thymidine; a set of six plates having media to which a volume of distilled water equal to that carrying the thymidine in the test plates acted as controls. After three days, one-half of all the plates were removed from the incubator, and the cells on them fixed and stained; after five and one-half days the remaining one-half were treated similarly. Ten colonies on each plate were randomly selected and the number of cells in each colony determined, so that the average number of cells per colony in a total of 30 colonies per treatment (3 plates x 10 colonies per plate was computed.

In experiments testing the effects of thymidine on nucleic acid metabolism, HeLa S3 cells in Eagles' medium were inoculated into T-30 flasks at a concentration of about 2.5 x 10^5 cells/flask. After three days incubation the media were removed from all flasks and to one-half of them a medium containing about 1 µc/ml H^3-uridine (2.3 c/mM, New England Nuclear) was added. To the other one-half was added the same medium but in which had been dissolved C^{14}-thymidine (2.4 mc/mM, New England Nuclear) in concentrated solution so as to not appreciably alter the

concentration of H^3-uridine in the medium. After 2 to 2-1/2 hours incubation, the media were removed and the cells extracted by the perchloric acid (PCA) method for separate analysis of the RNA and DNA, as described previously (2). The radioactivity of 0.2 ml of extract was determined by dissolving in a scintillation mixture and counting in a Packard Tricarb Scintillation Spectrometer. In samples with both C^{14} and H^3 in the extracts, the separate contributions of each isotope were determined with the aid of internal standards and by means of simultaneous equations (2). The relative amounts of nucleic acid were determined by measuring the optical density (O.D.) of the extracts at 267 mμ on a Beckman DU Spectrophotometer. In separate experiments we have found that specific activities (as counts per minute per ml per O.D. unit) determined by this method were at least as reliable as those using standard colorimetric determinations of RNA by the orcinol method and of DNA by the indole method (3).

RESULTS

The effects of unlabeled thymidine on the growth of HeLa S3 cells were unexpected. Thymidine presumably stimulates mitosis in mouse gut cells (4); it is included in many mammalian culture media; therefore, it was thought that thymidine would, if anything, stimulate growth. However, as Table I shows, the inclusion of this material in the medium always resulted in a decreased growth response. In the first experiment 200 μg/ml thymidine was included in the medium. Even though this is a

[illegible] high concentration ([illegible] millimolar), the strong inhibition of growth, amounting to more than 70 per cent was surprising.

The effects of 20, 100, 500 and 1000 µg/ml thymidine were compared and the results show that the inhibiting effect is dose dependent, and that the lowest concentration tested (20 µg/ml) significantly inhibited growth. In this experiment, Leighton tubes with cover slips were included and photomicrographs were made of the Giemsa stained preparations. Figure I shows the morphological effects of exogenous thymidine on HeLa S3 cells. The most obvious effect is cell enlargement, but there does not seem to be any differential increase in nuclear or cytoplasmic size

In an experiment comparing the effect of thymidine with other deoxynucleosides, thymidine at concentrations of 10, 50, and 100 µg/ml, and deoxycytidine, deoxyadenosine, and deoxyguanosine, each at a concentration of 100 µg/ml, were tested. All four deoxynucleosides together, at a concentration of 25 µg/ml each, were tested in the same culture. Again the inhibitory response of the cells to thymidine was dose dependent, with the concentration of 10 µg/ml causing a small but significant depression in cell count. The effect of 100 µg/ml was slightly less than in the previous experiment. All of the deoxynucleosides were inhibitory at 100 µg/ml but the effect of deoxycytidine and deoxyadenosine appeared to be not so great as that of thymidine, while the effect of deoxyguanosine was the same. The inclusion of equal amounts of the four nucleosides to make a final concentration of 100 µg/ml yielded results similar to those observed with either thymidine

or deoxyguanosine alone.

The final experiment of this kind again tested graded doses of thymidine and also the effect of three ribosides: adenosine at 100 μg/ml, cytidine at 50 μg/ml, and uridine at 10 μg/ml. Guanosine was not tested because of its low solubility. The results again demonstrated the effect of thymidine. The effect of 1 μg/ml thymidine is not significant, but that of 10 μg/ml is. Adenosine and cytidine did not appear to be as growth inhibitory as thymidine, but uridine, at 10 μg/ml, gave a response very similar to that of thymidine at the same concentration.

The results of the experiment testing the effects of thymidine on the average number of cells per colony are shown in Table II. Here 10 μg/ml does not appear to affect growth, and the effect of 20 μg/ml, although suggestive, is not significantly different from the control. The effects of 50 μg/ml and 100 μg/ml are significant, and the inhibitions agree remarkably well with hemocytometer data, especially since the two kinds of experiments were performed independently in laboratories on opposite sides of the USA.

The effects of thymidine in the medium on the uptake of H^3-uridine into DNA and RNA are surprising. Thymidine markedly inhibited the uptake of H^3-uridine into RNA (Table III). Since thymidine is not used in RNA synthesis, this result was rather disconcerting. Furthermore, even though an inhibitory effect of thymidine on the incorporation of H^3-uridine into DNA was expected, since its direct incorporation into DNA thymine would block uridine conversion to DNA thymine, the magnitude of

this effect with large amounts of thymidine was unexpected.

DISCUSSION

After the discovery by Reichard in 1951 (5) of thymidine as a specific precursor of deoxyribonucleic acid (DNA) and especially since its "rediscovery" by Friedkin (6), this compound has been used in so many studies on DNA metabolism that a list of the papers would require more space than that taken by this report. The greatest use of the compound has come about as the result of the preparation of tritium-labeled thymidine (H^3TdR), first accomplished by Hughes, in 1956 (7).

Even before its preparation, the potential radiation effects of H^3TdR were realized - indeed, the initial purpose for its preparation was in order to study the effect of radiation exclusively to the genetic material (8). In the last five years an appreciable number of reports have appeared, showing conclusively that H^3TdR does indeed exert radiobiologic effects as a result of its incorporation into DNA (9-12). Other reports of its adverse effects are somewhat arbitrary (13, 14) and one article, while pointing out a possible pitfall of H^3TdR, emphasizes its value in detecting a radiobiologic effect that could not have been noted with C^{14}TdR (15). Our own work, using the single cell plating technique with HeLa cells, has quantitatively measured the lethal effect of H^3TdR (16, 17). However, the work reported in the accompanying article (18) also shows that with short incubations, H^3TdR can successfully be used as a tracer for DNA metabolic studies without affecting viability.

One of the four basic requirements of radioactive tracers, as delineated by Kamen (19), is that, "abnormalities in metabolism must not be brought about through the action of the isotopic sample on the organism". It is quite obvious, from the results presented here, that C^{14}-thymidine, at concentrations often used in cell culture media, does not fulfill this requirement. One experiment in the accompanying report suggests that the inhibition of growth results in cell death. Even if cell death is not the inevitable result, the very marked decrease in cell counts and the morphologic response of cells exposed to thymidine, especially at levels of 100 μg/ml or more, make it clear that careful controls must be included in experiments using it in appreciable amounts. It is interesting to point out here that while it is necessary to use 100 μg/ml of the C^{14} labeled material to obtain 1 μc/ml, only 0.035 μg/ml of H^3TdR (6.7 c/mM, as now obtainable) is required to give the same activity.

The effects of exogenous thymidine on the incorporation of H^3-uridine into DNA and RNA appeared anomalous. However, during the preparation of these results for publication, the work of Morris and Fischer (20) and Morris, Reichard, and Fischer (21) was published. These workers, using a strain of murine mast cell neoplasm cells and a murine leukemic lymphoblast, showed that thymidine inhibited growth by suppressing the conversion of cytidylic acid to deoxycytidylic acid, thus depressing the rate of DNA synthesis. They also reported an inhibitory effect of thymidine on RNA synthesis. The inhibition of DNA synthesis in their studies was much

greater than the inhibition of RNA synthesis; in this respect out results are somewhat different since in two of our experiments the depressions of DNA and RNA synthesis were about the same. Nevertheless, our results confirm and extend those of these workers; undoubtedly the basis of the inhibitions in HeLa S3 cells are the same as in the cell systems studied by them. We have found that thymidine exerts similar growth inhibitory effects in a strain of Chinese Hamster cells (DFAF-33, kindly supplied to us by Dr. G. Yerganian), so that the phenomenon appears to be generally distributed among many kinds of cultured mammalian cells.

This metabolic defect does not, by itself, adequately explain the morphological response of HeLa S3 cells to thymidine. High concentrations of thymidine apparently inhibit the process of cell division and in this way are reminiscent of ionizing radiation in producing giant cells. Morris and Fischer (20) reported that the inhibition caused by thymidine in excess of about 10^{-3}M were not affected by deoxycytidine, and that the mechanism of inhibition by these concentrations was unknown.

SUMMARY

Experiments have been performed with C^{14} thymidine or unlabeled thymidine added to HeLa S3 cell cultures growing in Eagle's medium. The results show that: 1) Thymidine in the culture medium causes a concentration-dependent inhibition of growth, with effects being significant at levels as low as 10 μg/ml. 2) Thymidine in the medium reduces

the incorporation of H^3-uridine into both DNA and RNA. The effect on incorporation into RNA is unexpectedly great. 3) Other nucleosides, added singly or together to the medium, cause effects similar to those induced by thymidine.

The results are discussed in relation to the relative merits of C^{14} and H^3-labeled thymidine as tracers in metabolic and radiobiologic studies on the nucleic acids.

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TABLE I

THE EFFECT OF 3-DAY INCUBATIONS WITH THYMIDINE AND OTHER NUCLEOSIDES ON THE GROWTH OF HELA CELLS, MEASURED BY TOTAL CELL COUNTS IN HEMACYTOMETERS

Experiment No.	Nucleoside	µg/ml	Cell Count/ml ($\times 10^{-4}$)	% Depression
1	None	0(Control)	44.2 ± 1.8	-
	Thymidine	200	12.2 ± 0.7	72
2	None	0(Control)	112.2 ± 2.9	-
	Thymidine	20	81.5 ± 2.5	27
	"	100	25.0 ± 1.2	78
	"	500	17.3 ± 0.8	85
	"	1000	13.5 ± 1.2	88
3	None	0(Control)	76.9 ± 3.6	-
	Thymidine	10	63.5 ± 3.0	17
	"	50	50.4 ± 2.5	35
	"	100	31.1 ± 1.9	60
	Deoxycytidine	100	57.6 ± 3.7	25
	Deoxyadenosine	100	46.4 ± 5.2	40
	Deoxyguanosine	100	31.1 ± 2.7	60
	All 4	25 (each)	35.7 ± 3.1	54
4	None	0(Control)	75.6 ± 3.2	-
	Thymidine	1	67.9 ± 2.6	10
	"	10	58.5 ± 3.3	22
	"	100	28.2 ± 1.8	63
	"	1000	10.9 ± 1.0	86
	Uridine	10	61.1 ± 3.0	19
	Adenosine	100	53.2 ± 4.5	30
	Cytidine	50	64.7 ± 3.4	14

TABLE II

DEVELOPMENT OF COLONY SIZE AS A FUNCTION OF TIME OF GROWTH IN EAGLES' MEDIUM WITH VARYING CONCENTRATIONS OF THYMIDINE

Thymidine concentration µg/ml	Cells/colony after 3 days incubation	Cells/colony after 5-1/2 days incubation
0(Control)	9.0 ± 1.0	26.8 ± 3.3
10	8.7 ± 0.8	26.6 ± 2.8
20	6.9 ± 0.6	22.1 ± 3.0
50	6.3 ± 0.5	13.7 ± 1.7
100	4.8 ± 0.3	10.8 ± 1.6

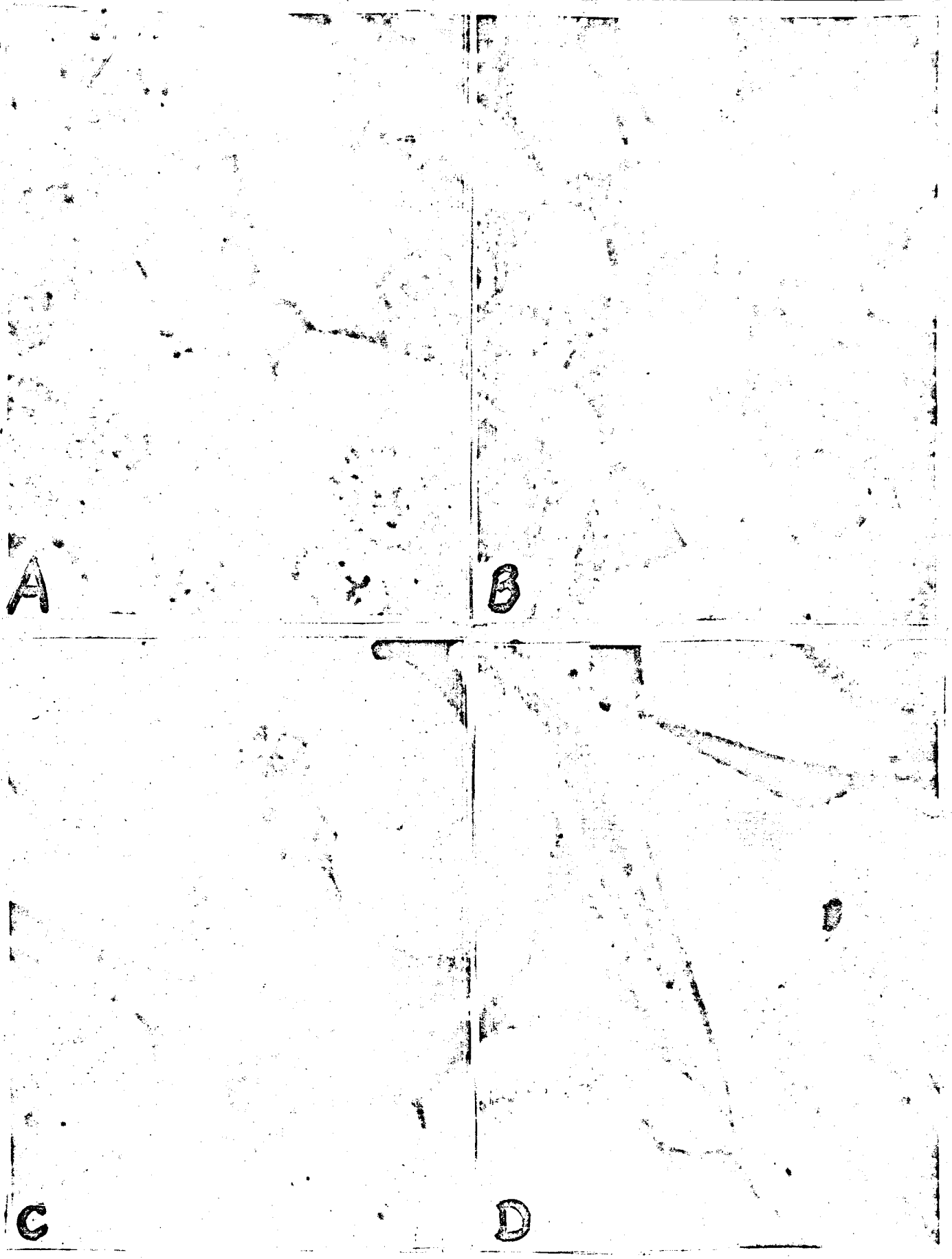
TABLE III

THE EFFECT OF EXOGENOUS THYMIDINE ON THE UPTAKE OF H^3-URIDINE INTO HELA S3 DNA AND RNA

Experiment No.	Addition to medium containing 0.1μg H^3 uridine	μg/ml	DNA Tritium cpm/μl/O.D.	% Change	RNA Tritium cpm/μl/O.D.	% Change
1	None	-	10.0	-	56.6	-
	C^{14}-Thymidine	340	0.9	-91	23.7	-58
2	None	-	6.3	-	41.1	-
	Unlabeled thymidine	200	3.2	-50	18.7	-55
	C^{14}-Thymidine	200	4.4	-31	20.4	-51
3	None	-	9.3	-	50.4	-
	Unlabeled thymidine	10	10.4	+12	49.3	- 2
	"	100	6.7	-28	38.0	-25
	"	1000	2.1	-77	11.4	-77

CAPTIONS

Figure 1. The effect of exogenous thymidine on the morphology of HeLa S3 cells. A - Control; B- 20 µg/ml thymidine. One of the mitotic cells appears abnormal, and some cells seem slightly enlarged, but most cells appear unaffected; C - 100 µg/ml thymidine. The cells are quite enlarged; D - 1000 µg/ml thymidine. The cells are large and cell stretching is obvious.



RADIATION RESEARCH, PAINTER, RASMUSSEN, and DREW, FIGURE 1